INDUCEMENT OF POLYPLOIDY IN THE INDIAN PEARL OYSTER, *Pinctada fucata* (GOULD)

DISSERTATION SUBMITTED BY

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NOVEMBER, 1998.

Dedicated to

My

Beloved Parents

DECLARATION

I hereby declare that this thesis entitled "**Inducement of polyploidy in the Indian pearl oyster, *Pinctada fucata* (Gould)**" *is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.*

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November, 1998.

CERTIFICATE

Certified that the dissertation entitled **"Inducement of polyploidy in the Indian pearl oyster *Pinctada fucata* (Gould)"** *is a bonafide record of work done by* **Mr. M. SAMAYA KANNAN**, *under our guidance at the Central Marine Fisheries Research Institute during the tenure of his M.F.Sc. (Mariculture) programme of 1997 – 1999 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.*

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सारांश

आनेवाले दिनों में भारत के मोती मुक्ता शुक्ति उद्योग में ट्रिपलायड (3N) उसर मोती मुक्ता शक्ति का एक महत्वपूर्ण भाग होगा, क्योंकि बीज रोपण में दीर्घकालिन परिपक्वता एक मुख्य प्रश्न है । इस अध्ययन में मोती मुक्ता शुक्ति **पिंक्टाडा फयकेटा** में पालीपलायड़ी उत्पादन का एक अच्छा तरीके का विकास उष्मिय आघात द्वारा किया गया । 10, 20 और 50 मिनट के बाद निशेचित बूण को लिया गया और जिसे 30, 32, 35, 38 एवं 6°C उष्मिय और शीतल आघात द्वारा 10 और 15 मिनट तक प्रभावित किया गया । पलायडी स्तर ट्रोकोफोर डिंभ के गुणसूत्र संख्या की जाँच द्वारा किया गया । इस परीक्षण द्वारा यह स्पष्ट हो गया है कि ट्रिपलायड़ी उत्पादन का अच्छा तकनिक निशेचित अंडों में पहले पोलार बाड़ी स्तंभन के लिए 35 उष्मिय प्रभाव के अंतरगत 10 मिनट और दूसरे पोलार बाड़ी स्तंभन के लिए उष्मिय प्रभाव 20 मिनट तक रखना है । जिससे क्रमशः 64.31 ± 2.66% और 74.31 ± 2.33% ट्रिपलायड बूण प्राप्त किया गया ।

50 मिनट पश्चात निशेचित अंडों को 35° C उष्मिय प्रभाव से प्रभावित किया गया तो ट्रेट्रापलायड (4N) बूण उत्पादन की प्रतिशतता अधिकतम (46.6 ± 1.66%) पाया गया । यहाँ पर नियंत्रित समूह में कोई बदलाव नहीं पाया गया । इसलिए इस परीक्षण द्वारा यह व्यक्त होता है कि ट्रिपलायड और ट्रेट्रापलायड मोती मुक्ता शुक्ति समूह उत्पादन के लिए उष्मिय आघात एक उत्तम एवं सस्ता तकनिक है ।

PREFACE

Bivalve molluscs such as edible oysters, pearl oysters, mussels, scallops, clams and cockles are widely distributed both in the tropical and temperate waters. Most bivalves are exploited for their delicious meat and as a cheap source of protein. In recent years, the depletion of wild stocks due to intensive exploitation and also poor productivity from the wild due to environmental pollution and damage, increased the demand for farm grown products. Unlike other bivalves, the pearl oysters are cultured for their precious pearls and shells.

The world aquaculture production of pearl oysters have been gradually decreasing for the past few years totalling 16,956 t in 1995 contributing nearly 0.33% of the total marine mollusc production of 5,093,747t (FAO,1997).

World production of pearls is estimated at between 80-100 tonnes/year. Japan is the largest producer of pearls and the largest importer of raw pearls too. Eventhough India had successfully produced cultured pearls in 1973 itself, it could not produce cultured pearls for world trade.

India supplied about 32 kg of marine pearls in 1989 forming only 0.49% of the total pearl imports of Japan estimated at 6,512 kg (Seamus McElroy, 1990).

The pearl quality may be improved by the application of modern tools such as genetic improvement and tissue culture (Alagarswami, 1987; Victor and Velayuthan, 1996). The application of genetic manipulations like polyploidy, gynogenesis and androgenesis in aquaculture has received considerable attention in recent years.

Triploids and tetraploids can be produced by application of thermal shock, pressure shock or chemical shock at just before meiosis and mitosis

of fertilized eggs respectively. Adult triploids are expected to be sterile since they can not synapse homologous chromosome during gametogenesis.

The advantages of induced polyploidy are :

i. Energy utilized for gamete production is diverted to somatic growth. Therefore, adult triploid could grow faster.

ii. Reduced gametogenesis increases the drymeat weight and prevents summer mortality which results in fetching good marketability throughout the year.

iii. Triploidy enhance the physiological activity like disease resistance etc.

iv. Tetraploid animals are used to produce cent percent viable triploids by crossing with normal diploids.

The induced triploid pearl oysters will be a useful tool for shortening the period of pearl culture, improving the yield and quality of pearl (Lin and Jiang, 1993).

Inducement of polyploidy in pearl oysters is not at present undertaken in India. As the species of pearl oysters are economically important in terms of production of natural and cultured pearls, the development of sterile oyter through polyploidy induction is of paramount importance in pearl production. During cultured pearl production, the oysters with matured gonad may hamper the process of nucleus implantation and lead to malformation of pearls.

Inducement of polyploidy may prevent gametogenesis which pave the way for large-scale production of high quality cultured pearls.

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ABSTRACT

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Samayakannan, M.,1998. Inducement of polyploidy in the Indian pearl oyster, *Pinctada fucata* (Gould). M.F.Sc. dissertation, Central Marine Fisheries Research Institute, C.I.F.E. (Deemed University), Kochi. (S.Dharmaraj).

In Indian pearl oyster industry, triploid sterile pearl oyster would play a vital role as prolonged maturation is one of the problems for nucleus implantation. In this present investigation, an effective method of inducing polyploidy in the Indian pearl oyster, *Pinctada fucata* (G.) developed by using thermal shock. The fertilized embryos were exposed to thermal shock at 30, 32, 35, 38 and 6°C at 10, 20 and 50 minutes after fertilization, for a duration of 10 and 15 minutes for heat shock and cold shock respectively. Ploidy levels were determined by chromosome counts from trochophore larvae. Triploid embryos were encountered in all treated groups. But no triploid or tetraploid embryos were recorded in the control groups.

It became explicit from the present study that the most effective procedure of inducing triploidy was exposing the fertilized eggs at 35°C at 10 min for the first polar body retention and at 20 min for the second polar body retention, which resulted in the formation of 64.3 ± 2.65 and 74.3 ± 2.33% triploid embryos respectively. When the fertilized eggs were treated at 35°C at the end of 50 min post fertilization, the highest percentage (46.6 ±. 1.66%) of tetraploid embryos could be recorded. Obviously, thermal shock appeared as the best low cost technology for the commercial production of triploid and tetraploid oyster larvae meant for pearl culture.

INTRODUCTION

1. INTRODUCTION

Pearl is one of the nine gems and the only gem obtained from the living organism. Since the dawn of human civilization, pearl has been an object of adoration. It has been treasured since long back.

Fishing for pearls has been practised for several centuries in many parts of the world including India. Occurrence of a natural spherical quality pearl from a wild oyster is a mere chance and it is not cost effective. The uncontrolled overfishing of these oysters led to the destruction of most of the natural pearl oyster beds known in Tamil as "*Paars*" of India. Moreover, the natural pearl oyster beds are often destroyed by the increasing number of bottom trawlers. Finally, the pearl fishery came to an end in India by 1961. Since then, the availability of natural pearl was found scarce and a need came to develop an alternative technology for the production of cultured pearls. Hence the Central Marine Fisheries Research Institute (CMFRI) had started pearl culture project in 1972.

In Indian waters, six species of pearl oysters are found, *viz.*, *Pinctada fucata* (Gould), *P. margaritifera* (Linnaeus), *P. chemnitzii* (Philippi), *P.sugillata* (Reeve), *P.anomioides* (Reeve) and *P. atropurpurea* (Dunker). Although several species of pearl oysters occur in the sea, only a few have been found to produce pearls of gem quality. The two species *P. fucata* and *P. margaritifera* standout distinct from other species in this respect. The Indian pearl oyster, *P. fucata* occurs in extensive beds in the Gulf of Mannar and to a lesser extent in the Gulf of Kutch. The black-lip pearl oyster, *P. margaritifera* confined mostly to Andaman and Nicobar waters are fished more for the shells than for their pearls.

In 1973, the C.M.F.R.I. had successfully developed an indigenous technology for the production of cultured pearls and made the breakthrough in the hatchery production of seeds of the Indian pearl oyster, *P.fucata* in 1981. The complete package of technology made the pearl culture industry to flourish in India.

In Indian waters, there are two spawnings of pearl oysters in a year, one in Jun-Sep. and the other in Nov-Feb. coinciding with south-west and north-east monsoon respectively. However in the Tuticorin Harbour farm, the inactive, maturing and mature gonads were found almost in all the months of the year (Chellam, 1987).

Normally, the pearl oysters with ripe gonad are not preferred for nucleus implantation as the gametes ooze out resulting in displacement or rejection of "nucleus" or "graft tissue" or both through the incision path. High mortality has been reported in the pearl oyster at the maturing and spawning season as in other bivalves (Wada *et al.*, 1989). Hence the commercial pearl production is always interrupted due to maturation. In this condition the development of triploid sterile pearl oyster could overcome this problem (Wada *et al.*, 1989; Lin and Jiang, 1993).

Adult triploids are usually sterile due to the inability of homologous chromosomes to synapse during meiosis which results in uneven or aborted separation of chromosome triplets. In such sterile animals, metabolic energy normally utilized for gonad development may be expected to be diverted into continued somatic growth (Stanley *et al.*, 1981). Sterility is advantageous in situations where the control of reproduction is desirable. Triploid bivalves exhibit reduced gonadogenesis and gametogenesis when compared to their

diploid equivalents and further more, induced triploidy is also expected to enhance physiological activity such as disease resistance (Beaumont and Fairbrother, 1991).

In molluscs, *cytochalasin* B has been used to induce polyploidy though its negative effects on survival have often been reported (Stanley *et al.*, 1981; and Tabarini, 1984).

Thermal shock, as it is a simple method, is employed in the inducement of polyploidy in bivalves.

In the present study, heat shock and cold shock were given to the embryos of the Indian pearl oyster, *Pinctada fucata* at their developmental stages. Inducement of polyploidy in *P. fucata* has got tremendous value in pearl culture. Any achievement in the inducement of polyploidy and its positive result would boost the pearl culture industry in the country. The study may facilitate the availability of sterile pearl oysters for pearl production throughout the year.

The major objectives of the present study were :

i. To investigate the optimal procedures *ie.*, shocking time and temperature for the induction of polyploidy.

ii. To compare the efficiency of heat shock and cold shock on ploidy induction and

iii. To prepare the metaphase chromosome spreads of the polyploid pearl oyster larvae.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Increasing interests in chromosomal engineering and induction of polyploidy in maricuture have often been reported as genetic improvement tools for successful production (Moav, 1976; Purdom, 1983). Polyploidy is one genetic manipulation like gynogenesis and androgenesis that might be helpful in aquaculture (Wilkins, 1981). Polyploidy is the condition of possessing more than twice the haploid number of chromosomes in the cell nucleus. A triploid animal possesses three rather than two copies of each chromosome and tetraploid having four copies in the cell nucleus.

The chromosome manipulation becomes feasible during the nuclear cycles of cell division and basically comprises the addition or subtraction of a complete haploid or diploid set in meiosis or mitosis respectively (Purdom, 1983). This may be achieved by treating the recently fertilized eggs either chemically with cytochalasin B (CB) or 6-Dimethyl Amino Purine (6-DMAP) or physically by applying thermal (heat or cold) shock or high pressure shock. Both the thermal and pressure shocks disrupt the formation of the metaphase spindles of the developing eggs and preventing replicated chromosome sets from separating into polar bodies at meiosis. But, CB inhibits micro-filament formation in cells (Maclean-Fletcher and Pollard, 1980). Thus it allows karyokinesis but prevents cytokinesis which results in a triploid zygote with a diploid female pronucleus and subsequent syngamy with a normal haploid male pronucleus.

It is not easy to arrest the first meiosis in most fishes, since this division takesplace in the ovary itself. The ploidy manipulation in crustacean

shellfish is also difficult, because they brood their eggs and embryos. Similarly, the European flat oysters, *Ostrea edulis* are even less amenable to ploidy manipulation.

In most vertebrates, eggs usually mature after completion of meiosis I whereas in invertebrates mature-eggs are arrested at prophase of meiosis I and only after fertilization or activation, the eggs complete meiosis I and II releasing two polar bodies. Thus the delayed meiosis in eggs of the Indian pearl oyster, *Pinctada fucata* provides a unique oppurtunity for ploidy manipulation by arresting both polar body I and II.

2.1. Inducement of polyploidy

Polyploidy in aquaculture was attempted first in the carp, *Cyprinus carpio* by Makino and Ojima (1943) and was followed by studies on many other fishes. The triploidy induction has been investigated for the molluscan aquaculture in America to market the American oyster, *Crassostrea virginica* (Gmelin) throughout the year (Stanley *et al.*,1981).

Induction of polyploidy has already been reported in many commercial bivalve molluscs by several authors (Stanley *et al.*, 1981; Tabarini,1984; Chaiton and Allen,1985; Downing *et al.*,1985; Allen *et al.*,1986; Yamamoto and Sugawara,1988; Uchimura *et al.*,1989; Wada *et al.*,1989; Gendreau and Grizel,1990 and Nell *et al.*, 1994). In India, research on polyploidy induction is limited to only in fishes. Though, India has vast resources of shellfishes, no ploidy work has been carried out in molluscs.

2.1.1. American oyster

The first attempt to induce triploidy in a bivalve mollusc, *Crassostrea virginica* was performed with CB by Stanley *et al.*(1981).

Stanley *et al.* (1984) observed that heterozygosity was higher in the meiosis I triploids produced by CB treatment during the first 15 min after fertilization.

The optimal concentration of CB in inducing triploid production in *C.virginica* was found to be 0.25 mg/l for 10-15 min (Barber *et al.*,1992). Allen and Bushek (1992) induced triploidy by inhibiting second polar body with 1mg CB/l from stripped gametes of *C. virginica.*

Scarpa *et al.* (1995) compared the efficiency of 6-DMAP and CB in inducing triploidy in the eastern oyster, *C.virginica* and they found that the efficiency was more in CB treatment.

The effectiveness of cytochalasin B on the ploidy and survival of D-stage larvae of *C. virginica* was studied by Supan *et al.*(1996). Anderson and Wallace (1996) induced triploidy in unconditioned *C. virginica*, using nitrous oxide at 7atm pressure for 15 min. and the success rate was found to be 56.8% by them.

2.1.2. Pacific oyster

The lower temperature between 18° and 20° C was found to be effective in inducing triploidy in *Crassostrea gigas* when CB was used as an inducing agent (Downing *et al.*,1985).

Chaiton and Allen (1985) induced triploidy in *C. gigas*, by means of pressure treatments which administered at 6000-8000 psi for 10 min duration at 10 min after fertilization and it resulted 57% triploidy. They also developed a technique to detect the ploidy level by flow cytometry.

Allen and Downing (1986, 1990) reported that the partial reproductive sterility observed in triploid oyster, *C. gigas* which greatly affected the reproductive physiology.

Application of thermal shock in inducing triploidy in *C. gigas* was studied by Quillet and Panelay (1986). They observed that the thermal shocks, applied at 10-15 min or 35-40 min post- fertilization at 35 and 38° C for 10 min yielded upto 25 and 45% triploid embryos respectively. They also obtained 60% triploid embryos when the treatment lasted for 20 min at same temperature.

Downing and Allen (1987) produced 88% triploidy in the Pacific oyster, *C. gigas* with CB treatment. Effect of CB on the induction of polyploidy in *C. gigas* has been studied by Guo *et al.*(1992). Shpigel *et al.* (1991) investigated the growth pattern, gametogenesis, physiology and biochemical composition of one year old diploid and triploid *C. gigas*.

The highest percentage(90%) of triploidy was obtained in *C. gigas* by Desrosiers *et al.* (1993) by treating the fertilized eggs with 300 µ*M* 6-DMAP at 15min after fertilization for 20 min.

Gerard *et al.* (1994a) attempted triploidy induction in *C.gigas* with 6-DMAP and obtained a mean of 85% triploids.

Guo *et al.*(1994) produced tetraploidy in *C.gigas* with heat shock by blocking mitosis I. They also produced upto 30% tetraploids by zygote-zygote fusion and plastomere fusion using polyethylene glycol (PEG).

Guo and Allen(1994) developed a unique procedure for the production of tetraploid *C. gigas* by inhibiting first polar body in eggs from triploids which was fertilized with normal haploid sperm.

Komaru *et al.* (1994) studied the ultrastructure of spermatozoa from induced triploid C. *gigas* that triploid sperm resembled diploid sperm and were significantly larger than that of diploid sperm.

All-triploid *C. gigas* were successfully produced by mating tetraploids with normal diploids (Guo *et al.*,1996).

2.1.3. Sydney rock oyster

Nell *et al.* (1994) studied the farming potential of triploid Sydney rock oyster, *Saccostrea commercialis* that triploid oysters could reach market size 6-18 months faster and maintained higher drymeat weight and higher condition index. Cox *et al.* (1996) studied the gonad development in diploid and triploid *S. commercialis*.

The application of CB for triploidy induction in *S. commercialis* resulted in greater survival and triploid percentage than 6-DMAP treatment (Nell *et al.*,1996).

Shoubai *et al.* (1994) produced tetraploid Jinjiang oyster, *C. rivularis* by heat shock, cold shock, chlorpromazinum and traditional chineae

medicine and they found that traditional chinese medicine yielded highest percentages.

2.1.4. Brooding oyster

The ploidy manipulation in brooding oyster is diffcult. However, Gendreau and Grizel (1990) induced triploidy and tetraploidy in European flat oyster, *Ostrea edulis* with CB and they obtained positive results.

In *Ostrea rivularis*, tetraploidy was induced by cold shock applied 3 min before the first cleavage gave 30 and 28% respectively (Shoubai *et al.*,1992).

Yu (1994) produced triploid *Ostrea gigas* with cold shock and further he observed that the larvae from treated groups had an obvious growth increase compared with control.

CB was found to be effective in inducing triploidy in *Ostrea cucullata* and gave higher percentage of triploidy than thermal shocks (Zeng *et al.*,1994).

Hawkins *et al.* (1994) studied the genetic and metabolic basis in meiosis I triploid *Ostrea edulis* treated with CB and they found that total dry tissue weight of meiosis I triploids was more than diploid siblings or meiosis II triploids due to increased heterozygosity in meiosis I triploids.

2.1.5. Scallop

Tabarini (1984) induced triploidy in the bay scallop, *Argopecten irradians* with CB and studied the effect of triploidy on growth and gametogenesis and he found that the adductor muscle weight and total body

tissue (wet) weight in triploid scallops were greater than diploid controls. He further observed that the majority of triploid scallops failed to mature.

The most effective CB dose to induce triploidy in *Pecten maximus* was 0.5 mg/l at 10min after fertilization for 20min which yielded 30% triploid embryos (Beaumont,1986). He also suggested that polar body counts were the simple method for assessing the degree of triploidy in embryos.

Komaru *et al.* (1988) produced triploid scallops, *Chlamys nobilis* with CB and detected the ploidy levels by microfluorometry.

Triploid black scallops, *Chlamys varia* were produced by treating with CB (Baron *et al.*,1989). They also found that there was no significant difference in growth between diploid controls and triploids, but mortality was higher for the triploids, before metamorphosis.

Canello *et al.* (1992) induced triploidy in the scallop, *Argopecten purpuratus* with heat shock and they analysed the post-fertilization time, temperature and duration of the thermal shock for obtaining highest percentages of triploidy.

Desrosiers *et al.* (1993) produced triploid giant sea scallop, *Placopecten magellanicus* with 6-DMAP and they obtained 95% triploid scallops. They also determined that increasing the treatment duration improved the efficiency of triploid induction, but prolonged incubations with 6-DMAP led to the development of abnormal larvae.

Winkler *et al.* (1993) induced triploidy in *Argopecten purpuratus* with CB and produced a mean proportion of 17% triploids and they also

obtained 6% triploids in the control groups which were treated with 0.005% Dimethyl sulfoxide (DMSO).

The heat shock of 31° C for 10 min applied at 10 min post- fertilization was found to be the optimal procedure for triploidy induction in *A. purpuratus* (Toro *et al.*,1995).

Zeng *et al.* (1995) found that the CB shock was more effective than thermal shocks in inducing triploidy in *Chlamys nobilis.* They also reported that the thermal shock treatment was an effective and simple method to induce triploidy from a commercial point of view.

In *Chlamys nobilis* triploidy was induced by treating the fertilized eggs with CB and cold shock in order to arrest first polar body and second polar body where the retention of second polar body gave higher incidence of triploidy (Lin *et al.*, 1995). He also studied the growth pattern of triploid scallop.

2.1.6. Mussel

Yamamoto and Sugawara (1988) applied heat shock and cold shock for triploid induction in the mussel, *Mytilus edulis* and obtained 97.4 and 85.3% triploidy repectively. they also found that longer heat shock treatment(20 min duration) improved the efficiency of triploid induction.

Beaumont and Kelly (1989) studied the larval growth of triploid *Mytilus edulis* which were produced by heat shock and CB treatment.

Electrofusion technique was proven to induce polyploidy in *Mytilus edulis* and *M. galloprovicialis* upto 36% triploids and 26% tetraploids when

electric pulses were applied prior to completion of the first cell division (Cadoret, 1992).

Scarpa *et al.* (1993) reported that tetraploids and pentaploids were produced in *M. galloprovicialis* by inhibiting both polar body I and II with a continuous CB treatment from 7-35 min after fertilization.

Desrosiers *et al.* (1993) produced triploid *M. edulis* with 6-DMAP and they found that the normal development in treated groups was low and the percentages of abnormal larvae were related to the duration of treatments.

Six different shocks like CB (1mg/l), heat (30° C), calcium (0.1*M*), caffeine (15m*M*), combined calcium-heat (0.1 at 30°C) and combined caffeine-heat (15m*M* at 30° C) shocks were compared in inducing triploidy in *Mytilus galloprovincialis* by Scarpa *et al.*,1994. They have reported that CB was the most effective in producing viable triploid individuals.

In *M. chilensis*, triploidy was induced with heat shock which yielded 51% triploid embryos and the triploid larvae showed difference in shell length than that of control (Toro and Sastre, 1995).

2.1.7. Clam

Allen *et al.* (1986) produced triploid soft-shell clam, *Mya arenaria* with CB treatment and studied the gametogenesis and sex ratio of the treated groups.

The chemical shock treatment of 0.5mg CB/l applied either from 0-15 min or from 15-30 min after fertilization gave the best results in triploidy

induction in the Manila clam, *Tapes semidecussatus* (Beaumont and Contaris, 1988).

Mason *et al.* (1988a & b) reported that the triploid *Mya arenaria* would grow larger and faster than their diploid siblings due to retarded gametogenesis and more heterozygous in triploids. In contrast, the triploid *Mercenaria mercenaria* treated with CB showed smaller dry tissue weight and all shell parameters than diploid controls over three growth seasons (Hidu *et al.*,1988).

Gosling and Nolan (1989) proved that thermal shock of 32°C for 10 min. yielded better results than CB treatment in triploidy induction in *Tapes semidecussatus*.

The effective CB treatment for the induction of triploidy in *Ruditapes philippinarum* was developed by Dufy and Diter (1990). In the same year, Diter and Dufy (1990) induced tetraploidy in embryos of *R. philippinarum* by treating the eggs with CB at 5 and 45 min after insemination. They also reported that no tetraploids were detected in 4 month-old spat.

Utting and Doyou (1992) studied the utilization of egg lipid reserves following induction of triploidy in *Tapes philippinarum* and they found that the percentage of triploids was higher for the embryos from brood stock fed on *Skeletonema* than from fed on *Dunaliella*.

Gerard *et al.* (1994b) obtained 95% triploid embryos in *Ruditapes decussatus* with 1mg CB/l applied at 15 min after fertilization for 20 min. In *T. philippinarum* application of 0.5mg CB/l yielded 70-77% triploid with 45% mean survival to the D-stage larvae (Utting and Child, 1994).

Triploidy was induced in fertilized eggs of *Tapes philippinarum* with CB at meiosis II. The growth rate, survival, food consumption, respiration rate and biochemical content of diploid and triploid larvae were studied for comparison and found to be similar (Laing and Utting, 1994).

Nell *et al.* (1995) induced triploidy in *T. dorsatus* with 1 mg CB/l for 15min exposure and further they observed that there was no difference in the growth rate of *diploid and triploid larvae.*

2.1.8. Pearl oyster

Induction of polyploidy has also been reported in pearl oysters by various authors. Uchimura *et al.* (1989) induced triploidy in the Japanese pearl oyster, *Pinctada fucata martensii* by inhibiting first polar body with 0.5mg *CB/l treatment.* The ploidy level was estimated by microfluorometry with 4',6-diamidino-2 phenylindole (DAPI) staining and found to be 65.4% triploidy.

Wada *et al.* (1989) produced 100% triploidy in *P. fucata martensii* by inhibiting second polar body with CB treatment and they found that cold shock(6.5° C) was also effective which gave 52% trploidy in the Japanese pearl oyster.

The effect of combination of caffeine and heat shock treatment in inducing triploidy in *P. fucata martensii* was investigated by Durand *et al.* (1990).

Shen *et al.*(1993) found that a hydrostatic pressure of 200-250 kg/cm at 5-7 min and 17-19 min post-fertilization for 10 min was most effective to induce triploidy in *P. martensii* which gave 76% triploids with high hatchability.

Jiang *et al.* (1993) produced triploid *P. martensii* with CB treatment and studied the growth of triploid pearl oyster. They observed that the growth rate of adult triploids were significantly higher than diploid siblings especially during reproductive seasons. They also suggested that the faster growth rate of triploid adults is caused by the retarded development of gonads in triploids.

Lin *et al.* (1996) observed that the mortality in the triploid *P.martensii* was higher during the developmental stages from D-stage to juvenile but, there was no difference in mortality between diploids and triploids during the stages of embryos and adults.

He *et al.* (1996) studied the gametogenesis, fertilization and development of the triploid *P. martensii* and they concluded that the triploid pearl oyters were sterile.

Lin *et al.* (1993) produced perfect pearls from triploid *P.martensii* with higher returns than from diploid pearl oysters. They also suggested that the induced triploid pearl oysters would be useful for shortening the period of pearl culture and producing good quality pearls.

Plate 1. The Indian pearl oyster, *Pinctada fucata* (G.) brood stock collected from natural pearl beds, Tuticorin.

DESCRIPTION OF SPECIES

ORDER	:	Dysodonta
FAMILY	:	Pteriidae
GENUS	:	*Pinctada*
SPECIES	:	*fucata* (Gould)
Common name	:	Indian pearl oyster.

Distinct characters

The hinge of oyster is fairly long and its ratio to the broadest width of the shell is about 0.85 and that to the dorsoventral measurement is about 0.76. The left valve is deeper than the right. Hinge teeth are present in both valves, one each at the anterior and posterior ends of the ligament. The anterior ear is larger than in the other species and the byssal notch, at the junction of the body of the shell and the ear, is slit-like. The posterior ear is fairly well developed. The outer surface of the shell valves is reddish or yellowish-brown with radiating rays of lighter colour. The nacreous layer is thick and has a bright golden-yellow metallic lustre.

Biology

Pearl oysters are sedentary animals found attached on dead corals, rocks and also on sand grits by means of byssus threads. They are filter feeders. They mainly feed on phytoplankters. They inhabit at depths from 10 to 20 metres.

In pearl oysters, the sexes are separate and they do not exhibit sexual dimorphism. *P. fucata* mature when they are 8 months old and about

17-25 mm size. They spawn in the ninth month coisiding with the monsoon seasons.

The longevity of a pearl oyster is about 5 to 6 years and the maximum attainable size is 100 mm.

Distribution in India

P. fucata are found in large numbers on "*Paars*" in the Gulf of Mannar which extend from Kilakarai to Kanyakumari and lesser extent in the Gulf of Kutch on intertidal reefs known as "*Khaddas*". In Palk Bay the pearl oysters are found on coarse sandy bottom.

Collection

Pearl oysters are collected by skin diving and SCUBA diving.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Spawning, Fertilization and Inducement of polyploidy

3.1.1.1. Collection of oysters

Samples of Indian pearl oyster, *Pinctada fucata* were collected from the natural pearl banks in the Gulf of Mannar, Tuticorin located at 08° 47'N; 78° 08'E. The oysters with ripe gonad were selected and cleaned to remove fouling organisms. Since the experiments were conducted in the natural spawning season, no conditioning was done prior to spawning.

3.1.1.2. Materials and Equipments

a. Knife to remove fouling organisms.
b. Perspex tank (30 litre capacity)
c. Thermometer
d. Jumothermometer
e. Immersion heater
f. Electronic thermostat
g. 30μm and 100μm sieves
h. Light microscope and embryo cup.
i. Beakers for gamete handling and larval rearing.

3.1.1.3. Chemicals

a) Neutral buffered formalin (4%)
b) Ethelene diamine tetra acetic acid (EDTA) for seawater treatment.

3.1.2. Ploidy verification

3.1.2.1. Materials and Equipments

a) A pair of droppers

b) Test tubes and pipettes to prepare solutions.

c) Screw capped vials of 5 ml capacity to store fixed embryos.

d) Insulated box and ice cubes for storage of fixed embryos.

e) Clean slides.

f) Slide warmer

g) Hot plate with magnetic stirrer.

h) Coplin jar for staining.

i) Filter papers.

j) Slide storing cabinet

k) Nikon Microscope with photomicrographic attachment.

3.1.2.2. Chemicals

a. Colchicine powders and solutions of strengths 0.02% for arresting of mitotic metaphase chromosomes.

b. Seawater and distilled water as hypotonic solution.

c. Glacial acetic acid and methanol to prepare Carnoy's fixative.

d. 50% Acetic acid.

e. Giemsa stain stock solution.

f. Di-sodium hydrogen phosphate and potassium di-hydrogen phosphate to prepare Sorenson's phosphate buffer (pH 7.0).

3.2. Methods

3.2.1. Spawning and fertilization

Spawning in pearl oysters was induced by thermal stimulation. The oysters were placed in 30 litres of filtered seawater taken in a perspex tank with the size 50cm x 30cm x 30 cm. The water temperature was raised from the ambient temperature of 28 to 30° C using an immersion heater. The required temperature was set with the help of Jumothermometer. Aeration was provided during the process in order to disperse the heat uniformly in the tank. The immersion heater and Jumothermometer were connected to an automatic thermostat.

If the oysters were not spawned at 30° C, the water temperature was further raised to 32° C. During the process of gradual heating, the oysters were induced to spawn. Once the oysters started spawning, they were individually isolated in a separate beaker and allowed to continue the spawning till the gametes were released fully.

The eggs of three or four females were pooled together and sieved through 100 μm nylebolt cloth. Large dust, faecal matters and broken pieces of byssus threads were retained in the cloth. The eggs alone passed through the mesh. The eggs thus collected were kept for fertilization in 1litre of filtered seawater.

Similarly, the sperm were also collected by passing through 30 μm sieve. Eggs and sperm were kept separately at 24± 1° C before fertilization to synchronize the egg development.

The time, when the sperm were added to the suspension of eggs was considered to be the start of the experiment. The eggs and sperm were gently mixed for proper fertilization. After five minutes, the eggs-sperm suspension was made upto 5 litre with isothermal filtered seawater and divided into ten aliquots of 500 ml each. The embryos were maintained at the ambient temperature of 24±1° C till they were given thermal shock.

3.2.2. Inducement of polyploidy

Embryos ($5x10^4$) of each aliquot were kept in a PVC pipe (6cm dia; 9cm hight) fixed with 30 μm sieve cloth at one end. The embryos were rinsed with filtered seawater to remove excess sperm. Inducement of polyploidy was done by immersing the sieve containing the embryos in heated or chilled seawater.

3.2.2.1. Experiment 1: effect of timing of initiation on polyploidy inducement

To investigate the effect of timing of initiation on polyploidy inducement, each aliquot was treated one by one at 30° C at 10, 15, 20,...and so on upto 50 min after fertilization with an interval of 5 min. The duration of each treatment lasted for 10 min. The remaining one was untreated and kept as control. At every 5 min. of post- fertilization, a sample of embryos was taken from the control and fixed in 4% neutral buffered formalin. Polar body extrusion time was estimated from the microscopic observations of the fixed embryos.

3.2.2.2. Experiment 2: effect of heat shock on polyploidy inducement

Based on the results obtained from Experiment-1, three different temperature values 32, 35 and 38 ±0.5° C were investigated. The embryos were divided into four aliquots and treated at 10, 20 and 50 min post-fertilization

Plate 2. A view of experimental setup for inducing polyploidy by heat shock.

Plate 3. The fertilized embryos under heat shock treatment. (A close view).

Plate 4. Cold shock treatment of developing embryos by using ice.

Plate 5. Rearing of larvae after thermal shock treatments.

for 10 min duration in order to block meiosis I, meiosis II and first cleavage respectively. Remaining one was kept as control.

3.2.2.3 Experiment 3: effect of cold shock on polyploidy inducement

Similar to Experiment-2, the embryos were also treated at lower temperature of 6° C (±1°C) at 10, 20 and 50 min after fertilization for 15 min duration. The untreated one was kept as control.

3.2.3. Larval rearing

After treatment, embryos were reared in 1 litre of filtered seawater, maintained at 23±1°C. Seawater for all treatments and larval rearing was filtered and treated with 1 mg/l ethylene diamine tetra acetic acid (EDTA) as a precaution against metal contamination (Utting and Helm, 1985).

3.2.4. Ploidy verification

Ploidy level of all treated groups as well as control was determined by the partially modified direct chromosome counts methodology of Klingerman and Bloom (1977). The trochophore stage was considered to be the best stage for direct chromosome counts. Hence,10 hrs after fertilization, a portion of free swimming larvae were collected from each group and treated with 0.02% colchicine for 2 hrs. Then they were transferred to a hypotonic solution (1 part seawater+ 2 parts distilled water) for 30 min. Before fixation, Cornoy's fixative was added at 1:10 ratio to the hypotonic solution to prevent cells bursting upon sudden exposure to full-strength Cornoy's fixative. Subsequently, the larvae were fixed with fresh Cornoy's fixative (3 absolute methanol : 1 glacial acetic acid) for 2 hrs at 4°C. During fixation, the fixative was changed 3-4 times. Later, the larvae were taken out and excess fixative was removed as far as possible. The fixed larvae were dissociated in 50% acetic

acid immediately. The suspension of larvae was pipetted out onto a clean, pre-warmed (45°C) microslides. Then the slides were air-dried and stained in 5% Giemsa (5ml Giemsa stock + 95ml Sorenson's phosphate buffer) at pH 7.0 for 20 min.

The clear metaphase spreads were only counted. The percentage of ploidy level of each group was estimated by the frequency distribution method (Wada *et al.*,1989). For each treatment 25-35 metaphase spreads were counted.

The chromosome number (n=14, 2n=28) of *P. fucata* has already been reported and conformed by Alagarswami and Sreenivasan (1987). Hence, the ploidy level was described as following: chromosome counts in the range 26-28 = diploid(2n); 39-42= triploid(3n) and 52-56 = tetraploid(4n).

3.2.5. Data analysis

The effect of thermal shock on polyploidy induction was determined using analysis of variance test and students' t-test analysis were adopted to test the differences between treatments.

RESULTS

4. RESULTS

4.1. Spawning and fertilization

Pearl oysters were spawned at 34 ± 1° C and the response was noticed first in male oysters followed by females. The addition of stripped sperm to the spawning medium gave better spawning results. The fertilized embryos were also obtained by "stripped gametes" where the time of fertilization is controllable (Allen and Bushek, 1992). By following this method the percentages of undeveloped embryos were found in higher numbers. Hence, to obtain gametes, thermal stimulation was carried out in all the experiments and the fertilization rate was recorded as more than 95%.

4.2. Experiment 1: effect of timing of initiation on polyploidy inducement by heat shock

The fixed embryos were observed for polar body extrusion under light microscope (20x). Most of the eggs completed the first meiosis at 15 minutes after fertilization at 24±1°C. The second meiosis and first cleavage were found after 25 minutes and 55 minutes respectively after fertilization at 24±1°C (Table 1).

The heat shock at 30°C was given to induce polyploidy. The percengage of aneuploidy was observed in low percentages in all treated groups. Triploidy was noticed in all the treated groups (Table 2) ranged from 10.3 ± 1.45 to 31.0 ± 2.08%. In the control groups triploids and tetraploids were not found. The highest percentage of triploidy was obtained in treatment group 1 and 3 which was corresponding to extrusion of first and second polar bodies. The percentage of triploidy (31%) in the treatment group 3 (20 minutes after fertilization) was significantly higher ($F_{(8,18)}$= 13.9736, P< 0.01) than that of

Plate 6. Metaphase chromosome spreads from diploid embryos of *P. fucata*

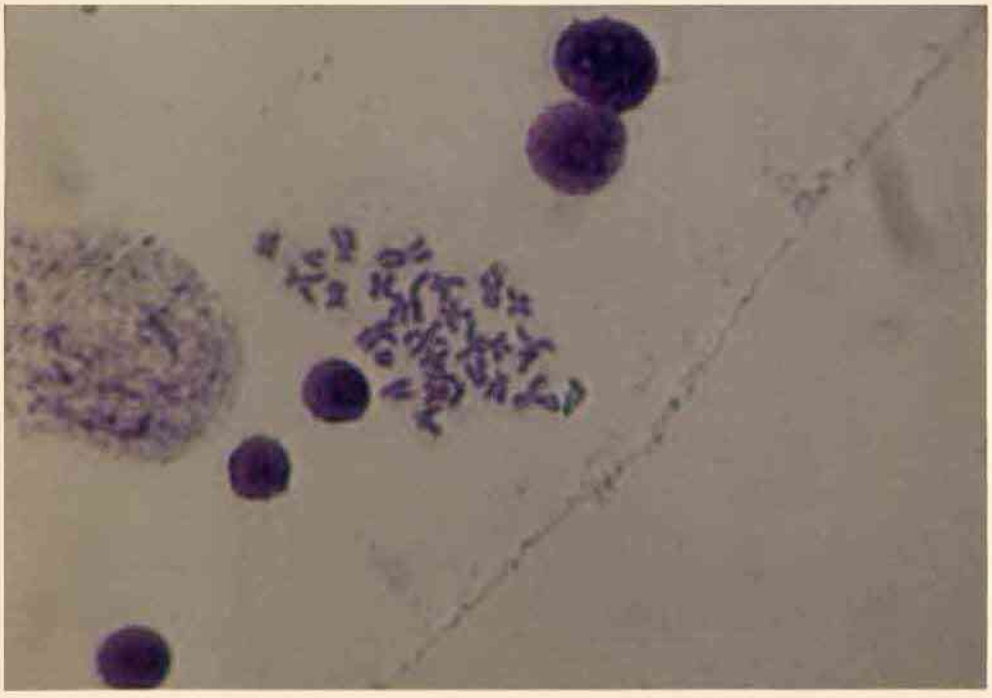

Plate 7. Metaphase chromosome spreads form triploid embryos of *P. fucata*

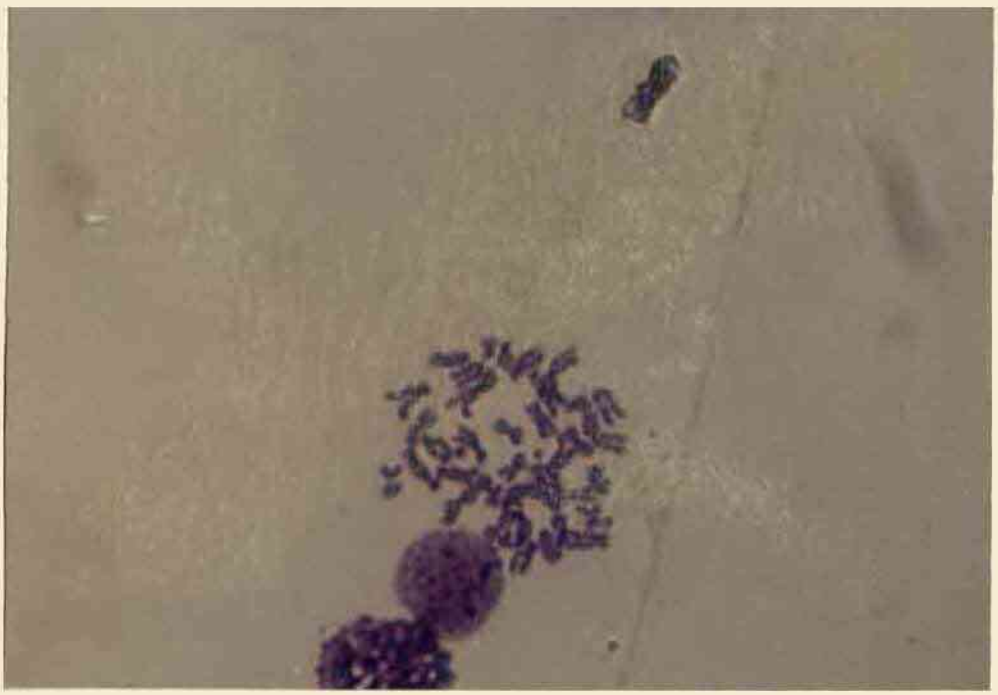

Plate 8. Metaphase chromosome spreads form tetraploid embryos of *P. fucata*

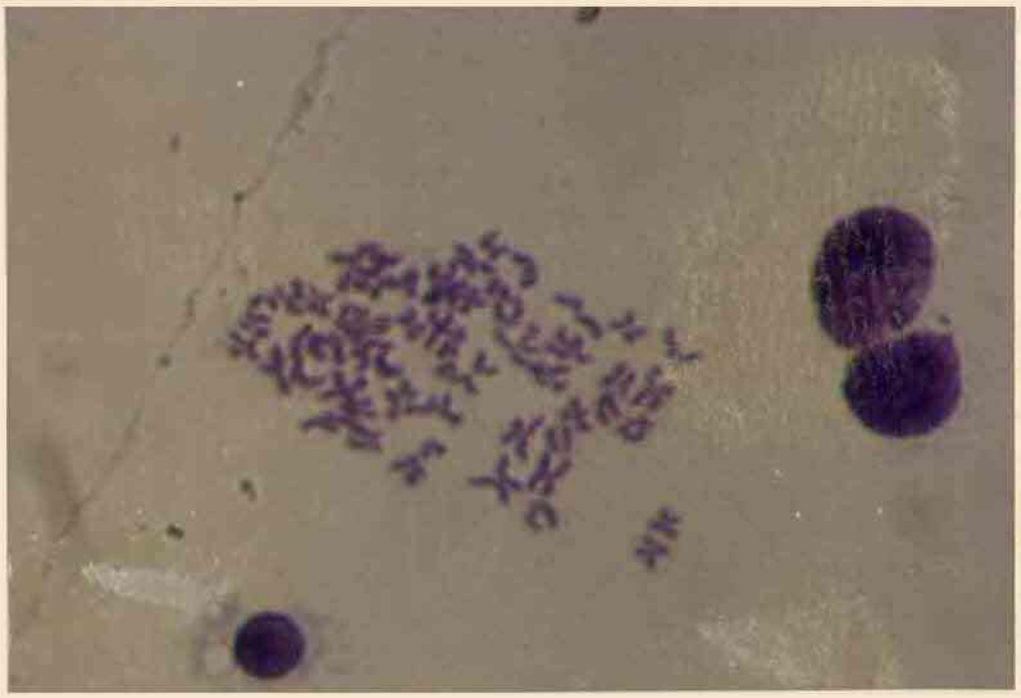

Table 1. The Percentages of untreated eggs with polar body I (PBI), polar body II (PB II) and 2-cell stage (Experiment 1).

Time after Fertilization (min)	PB I (%)	PB II (%)	2 – cell stage (%)	Total Number of eggs observed
5	0±0.00	-	-	50
10	25±1.15	-	-	75
15	64±1.73	0±0.00	-	70
20	73±3.51	26.3±3.28	-	69
25	-	71.0±1.15	-	90
30	-	81.0±0.57	-	53
35	-	85.6±1.76	-	47
40	-	83.6±2.33	-	50
45	-	-	0±0.00	65
50	-	-	18.6±2.33	74
55	-	-	52.0±1.53	68
60	-	-	70.3±1.45	70

Values are mean ± SE (n=3)

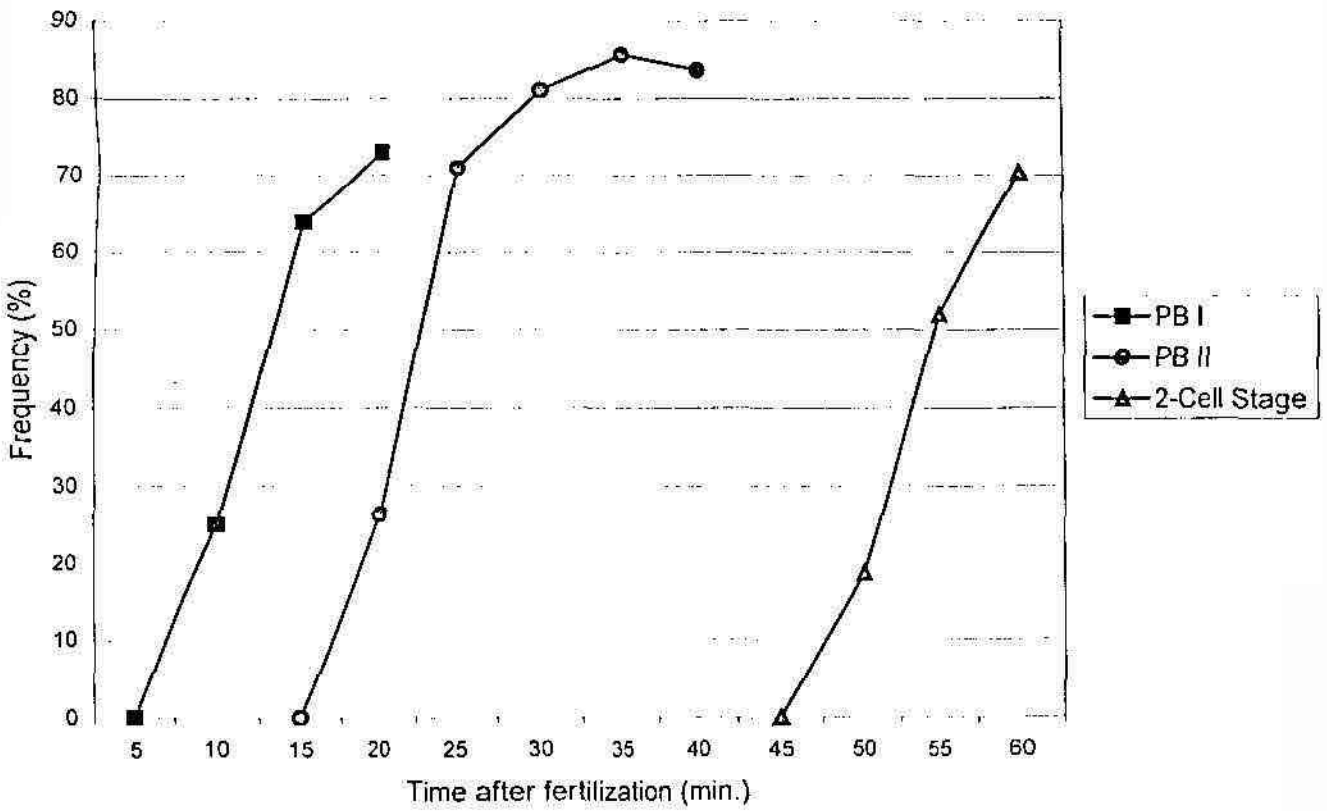

Fig. 1. Timing of PB I and PB II extrusions and 2-cell stage from fertilized eggs in experiment 1

Table 2. Effect of initiation time on polyploidy inducement at 30°C (Experiment 1).

Treatment groups	Initiation time of Treatment (min)	Diploidy (%)	Triploidy (%)	Tetraploidy (%)
1	10	74.0±2.64	29.3±2.18	-
2	15	86.0±1.53	14.0±1.52	-
3	20	68.0±1.73	31.0±2.08	1.0±0.57
4	25	82.0±2.88	15.3±1.45	2.6±1.45
5	30	76.6±1.45	18.6±1.45	4.6±1.45
6	35	79.3±2.73	14.3±1.45	6.3±1.76
7	40	78.6±2.40	11.3±1.85	10.0±0.58
8	45	76.0±1.53	10.3±1.45	13.6±0.88
9	50	60.3±3.93	18.3±3.53	21.3±1.85
10	Control	100.0±0.00	-	-

Values are mean ± SE (n=3)

Table 3. ANOVA Table showing the significant variation between the initiation time and triploidy percentages

Source of variation	df	SS	MS	F
Treatment [a]	8	1333.1851	166.6481	F=13.9736**
Error	18	214.6667	11.9259	
Total	26	1547.8518		

[a] - Time.

** - Significance at 1% level

Table 4. ANOVA table showing the significant difference between initiation time and induced tetraploidy percentage

Source of Variation	df	SS	MS	F
Treatment [b]	6	909.9047	151.6507	F=28.95**
Error	14	73.3333	5.2381	
Total	20	983.2380		

[b] - Time.

** - Significance at 1% level

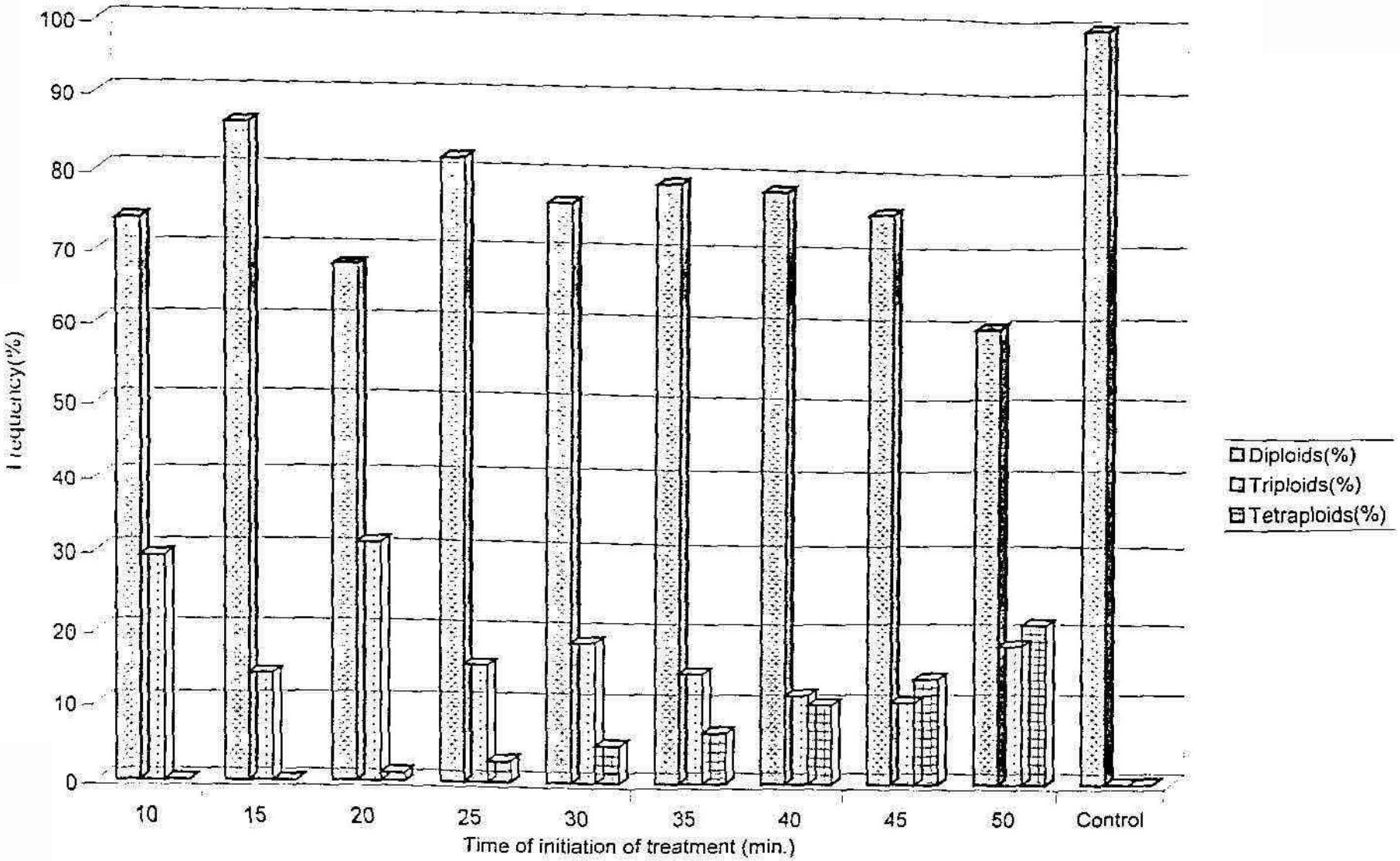

Fig. 2. Effect of timing of initiation on polyploidy inducement at 30°C (Experiment 1)

all other treatments (Table 3) except the treatment group 1 (10 minutes after fertilization, 29.3% triploids). There was no significant difference ($t = 0.55$, $p>0.05$) between treatment groups one and three.

The highest percentage (21.3± 1.85%) of tetraploidy was obtained from treatment group 9 (50 minutes after fertilization), which was highly significant ($F_{(6,14)} = 28.95$, $P< 0.01$) than other treatment groups (Table 4).

4.3. Experiment 2 and 3: effect of heat shock and cold shock on polyploidy inducement.

Five temperature shock treatments were employed for producing triploidy and tetraploidy (6,30,32,35 and 38°C). The highest percentage of triploidy (74.3 ± 2.33%) was found at 35°C treatment group which was significantly higher ($F_{(4,8)} = 5.2118$, $p<0.05$) than other treatment groups except 38°C (64.6%). The triploidy percentages 31.0%, 37.0 % and 49.3% at 30°C, 32°C and 6°C were noticed respectively (Table 6). There was no significant difference ($P>0.05$) between groups treated at 35 and 38°C. Despite insignificant difference observed between the triploidy at 38°C treated groups and 35°C, abnormal larvae were observed in higher numbers in 38°C group.

The highest percentage of tetraploidy (46.6%) was noticed (Table 6) at 35°C temperature which was significantly higher ($F_{(4,10)} = 17.7883$, $P<0.01$) than other treatment groups (Table 7) except the groups treated at 6 and 38°C. The abnormal larval percentage was higher in tetraploidy groups treated at 38°C like triploids.

Table 5. Percentages of diploidy, triploidy and tetraploidy induced at different temperatures.

Treatment	Temperature (°C)	Initiation Time of Treatment (min)	Duration (min)	Diploidy (%)	Triploidy (%)	Tetraploidy (%)
1[a]	6	10	15	50.6±3.48	49.3±3.48	-
		20	15	61.6±2.03	37.6±2.02	1.0±0.57
		50	15	45.0±2.64	14.3±0.88	40.6±3.38
		C	-	100±0.00	-	-
2[b]	30	10	10	74.0±2.64	26.0±2.64	----
		20	10	68.0±1.73	31.0±2.08	1.0±0.58
		50	10	60.3±3.93	18.3±3.52	21.3±1.85
		C	---	100.0±0.00	----	----
3.	32	10	10	69.0±2.08	31.0±2.08	----
		20	10	63.0±2.31	37.0±2.31	----
		50	10	50.3±4.37	18.3±2.02	31.3±2.73
		C	---	100.0±0.00	----	----
4.	35	10	10	35.6±2.96	64.3±2.96	----
		20	10	21.3±3.84	74.3±2.33	4.3±1.76
		50	10	32.6±1.73	20.6±2.40	46.6±1.66
		C	---	100±0.00	----	----
5.	38	10	10	36.0±2.08	64.0±2.08	-
		20	10	25.0±2.51	64.6±2.60	10.3±0.88
		50	10	28.3±4.41	27.6±3.38	44.0±2.31
		C	-	100±0.00	-	-

Values are mean + SE (n=3)

[a] - data obtained from experiment 3
[b] – data obtained from experiment 1

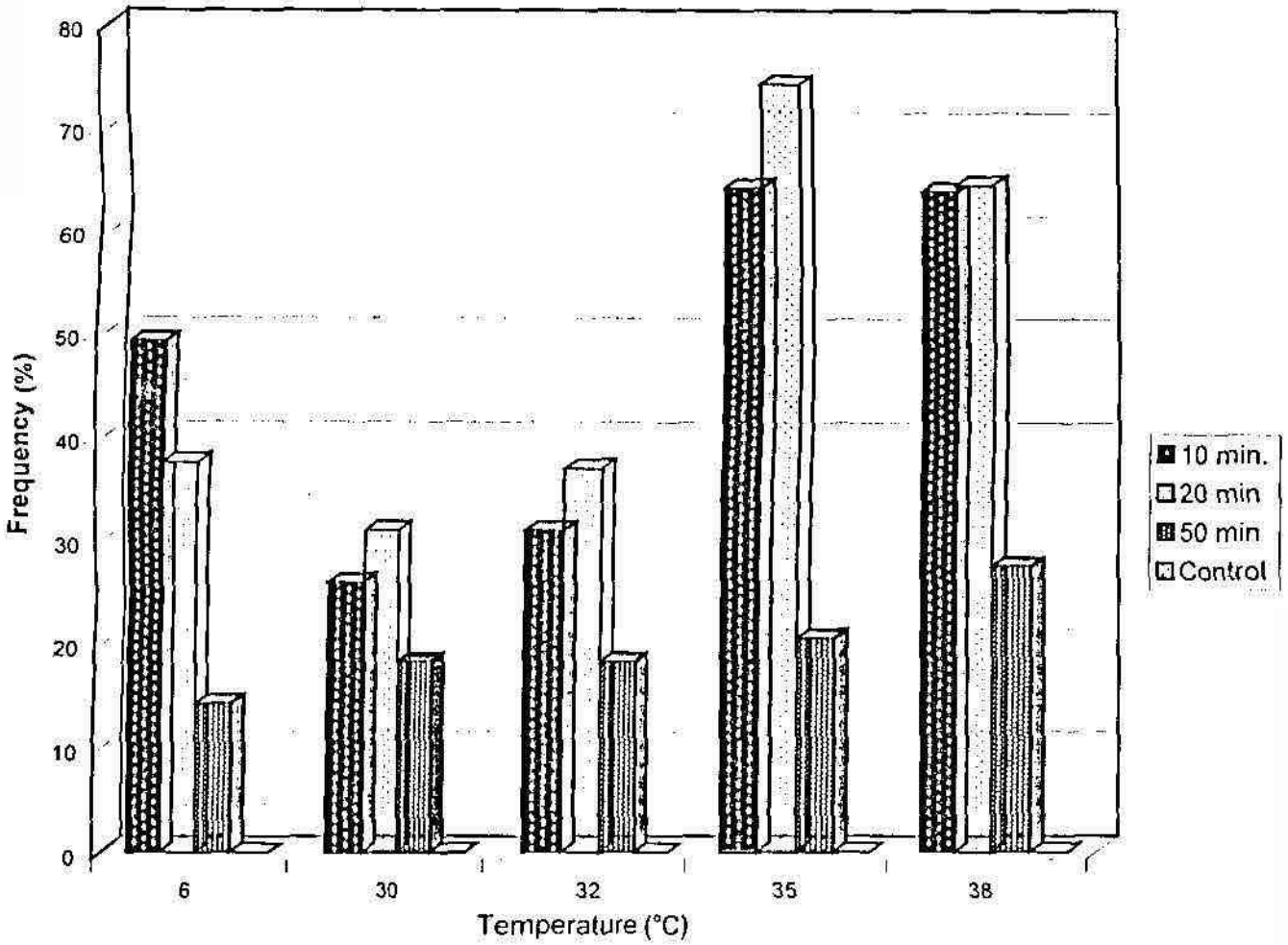

Fig. 3. Effect of thermal shock on triploidy induction at different treatment times

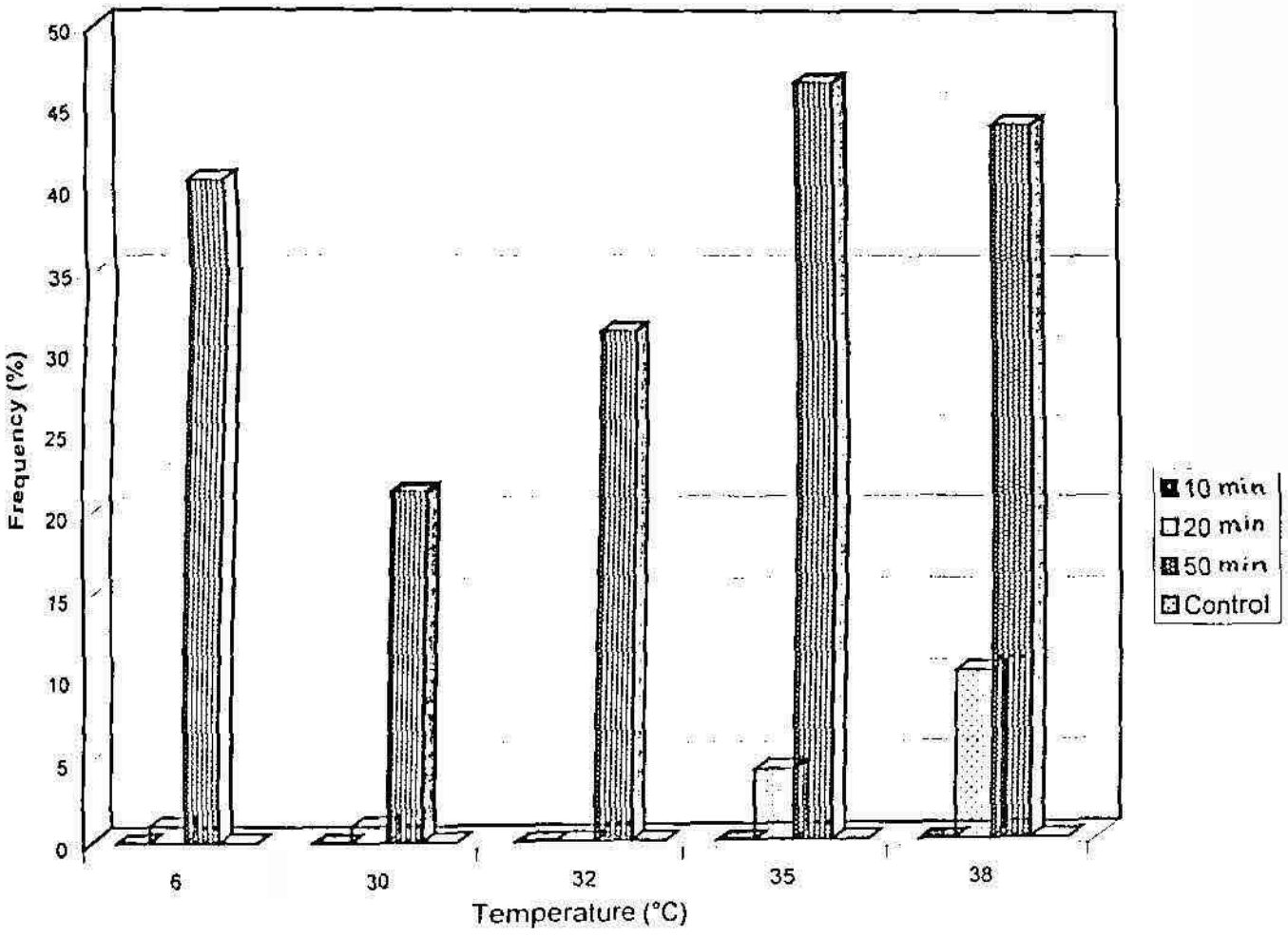

Fig. 4. Effect of thermal shock on tetraploidy induction at different treatment times

Table 6. ANOVA table showing the effect of temperature in inducing triploidy at 10,20 and 50 min. after fertilization

Source of variation	df	SS	MSS	F
[1]Sample	2	2637.504	1318.752	F =13.2130**
[2]Treatment 2	4	2080.716	520.179	F = 5.2118*
Error	8	798.456	99.807	
Total	14	5516.676		

[1] - Time of initiation of Treatment after fertilization
[2] - Temperature of the treatment
** - Significance at 1% level
* - Significance at 5% level

Table 7. ANOVA table showing the effect of temperature in inducing tetraploidy at 50 min after fertilization

Source of variation	df	SS	MS	F
Treatment[1]	4	1299.7333	324.9333	F = 17.7883**
Error	10	182.6666	18.2666	
Total	14	1482.3999		

[1] - Temperature of the treatment
** - Highly significant at 1% level

4.4. Comparison of the effect of heat shock and cold shock for triploidy and tetraploidy inducement.

From the results obtained (Experiment 2 and 3) the overall triploidy percentage was comparatively lower in cold shock than that of heat shock at 35 and 38°C and higher in cold shock than heat shock at 30 and 32°C treated groups.

Unlike triploids in tetraploidy there was no significant difference ($t = 1.59$, $P>0.05$) between 6 and 35°C groups treated at 50 min after fertilization. Triploidy percentage obtained from 35°C groups treated at 10 and 20 min after fertilization were highly significant ($t=6.161$, $P<0.05$) than triploids produced by cold shock at the same time treatment. For tetraploidy percentage, there was no significant difference ($t=1.59$, $P>0.05$) between 35 and 6°C groups treated at 50 min after fertilization.

DISCUSSION

5. DISCUSSION

The present work was carried out with the main aim of optimizing the thermal shock procedure for inducing triploid and tetraploid pearl oyster embryos which would be helpful to boost the Indian pearl oyster industry for world trade.

5.1. Spawning and fertilization:

As the pearl oysters readily respond to the thermal stimuli (Dharmaraj *et al.*, 1991) , the gametes were obtained for all experiments through thermal stimulation and the fertilization rate was also more than 95%. Although 'strip' spawning is the recommended method for obtaining eggs for triploidy induction (Allen and Bushek, 1992) but it was observed that the undeveloped embryos were found in higher numbers. The reason for this may be that even the 'damaged' or 'maturing' oozytes are activated, and do not develop further. Nell *et al.*(1996) reported that there was much greater variability in the speed of egg development in 'stripped' eggs than in those from natural spawning and strip spawning in Sydney rock oysters produced fewer viable larvae than natural spawning.

Synchronous development of eggs and timing of treatment are two critical factors for producing high percentages of triploid embryos (Wada *et al.*,1989). In some cases, uncontrolled fertilization or other factors have led to non-synchronous development (Beaumont,1986; Gosling and Nolan,1989; Durand *et al.*,1990) and therefore low percentages of triploids. The development of eggs used to vary depending on the egg size (Nell *et al.*,1996) and the percentage of triploids will vary according to the availability of egg lipid resources (Utting and Doyou,1992).

It is already known that CB affects micro-filament formation (Maclean-Fletcher and Pollard,1980; Schatten and Schatten, 1981; and Yahara *et al.*,1982) which inhibits cytogenesis but not karyokinesis (Carter,1967) whereas physical shocks like pressure, cold, heat and caffeine shocks disrupt microtubules (Yamamoto *et al.*,1990). Hence, the application of thermal shock should be given during meiosis I, meiosis II or first cleavage to produce high percentages of polyploid larvae. In the present study, the development of fertilized eggs was studied and the synchronous development was observed to be high. Extrusion time of the first and second polar body in *P. fucata* (G.) was similar to that reported for *P. fucata martensii* and most of the eggs had extruded the first and second polar body by 15 and 25 min after fertilization at 24±1°C (Table1) compared to 15 and 24 min at 23°C for *P. fucata martensii* (Komaru *et al.*, 1990a). Similar results were obtained by Komaru *et al.* (1990b) that most of eggs at 25°C completed their first and second meiosis at 15 and 27 min after insemination. In *Saccostrea commercials*, 50% of the eggs had released their first polar body between 17 and 19 min post-fertilization at 25°C compared to between 15 and 20 min at the same temperature for *Crassostrea gigas* (Guo *et al.*,1992a & b; Desrosiers *et al.*, 1993; Longo *et al.*, 1993 and Nell *et al.*, 1996). As for *C. gigas*, maximum triploidy induction corresponded to exposure to CB when approximately 50% of eggs had released the first polar body (Allen *et al.*,1989).In the experiment 1, 29.3% triploidy embryos were induced when 25% of eggs had released their first polar body were as 31% triploidy were induced when 26.3% of eggs released their II polar body.

Results from the experiment 1 (Table 1and 2) show the variation in timing of meiotic divisions (Fig.1and 2) between different batches of eggs, thus emphasizing the importance of using development criterion *i.e.* polar body extrusion time, rather than elapsed time to measure treatment initiation. Although polar body counts was the simple method for assessing the degree of triploidy in embryos, polar body counts may not accurately indicate the future representation of ploidy levels due to differential mortality (Beaumont,1986). Inaccuracy may arise also, if polar bodies are difficult to distinguish (Beaumont and Contaris, 1988) or are dislodged during sampling or preparation; this may explain partially the large proportion of eggs having no polar bodies (Scarpa *et al.*, 1994). Hence, the ploidy levels of each treated group was determined by direct chromosome counts (Beaumont and Kelly, 1989).

Diploid individuals of *P. fucata* had a chromosome complement of 2N=28 while triploids (3N) and tetraploids (4N) had 42 and 56 chromosome numbers respectively (Plate 6, 7 & 8). These results corroborate the previous report of Alagarswami and Sreenivasan (1987) for this species. Komaru and Wada (1985) had reported the diploid chromosome number (2N = 28) for *P.fucata martensii* and in *P. imbricata*, Wada (1978) had recorded the same numbers. The chromosomal information on *P. fucata* would be useful in monitoring polyploidization and other kinds of chromosome-set manipulation in this species.

5.2. Triploid inducement

The results of triploid inducement recorded in this investigation indicated that all the treatments were effective in producing triploid embryos in *P. fucata*, reconfirming the effectiveness of heat shock to induce triploidy in

bivalve molluscs (Quillet and Panelay, 1986, Wada, *et al.*, 1989 and Scarpa *et al.*, 1994). The actual difference between normal incubation temperature and the thermal shock temperature is likely to be of more importance than the actual temperature of the treatment. An upward difference of 11°C had proved capable of inducing. "chromosome-doubling" during the meiotic divisions (Beaumont and Fairbrother, 1991). Thermal shock has been reported to induce high amounts of triploidy in the blue mussel, *M. edulis* (Yamamoto and Sugawara, 1988; Beaumont and Kelly, 1989). Aneuploids were observed in all treated groups similar to those experiments done by Yamamoto and sugawara (1988), Beaumont and Kelly (1989) and Nell *et al.* (1996). No polyploids could be encountered in the controls of any treatment as already reported by Gosling and Nolan (1989). However, Yamamoto and Sugawara (1988), Winkler *et al.* (1993) and Scarpa *et al.* (1994) observed triploids in control groups due to handling stress or some other factors

The optimal procedure for triploid induction was the heat shock (35°C for 10 min) applied 10 min after fertilization (meiosis I) and 20 min after fertilization (meiosis II) (Table 4). This corresponds to the beginning of anaphase of meiosis I and II (Komaru *et al* ., 1990 a & b). Triploid pearl oysters obtained by the treatments started 5 min after insemination may be induced by preventing the extrusion of the first polar body; those obtained by the treatments from 15 to 20 min after insemination may be induced by second polar body retention (Wada *et al* ., 1989). In the present study, the percentages of triploidy (26.0-64.3%) obtained by blocking meiosis I using heat shock were comparable to those reported for other bivalves, *C. gigas* (25-45%, Quillet and Panelay, 1986), *T.semidecussatus* (23.1-55.6%, Gosling and Nolan, 1989), *M. edulis* (25%, Beaumont and Kelly, 1989), *O. cucullata* (4.1-55.2%, Zeng *et al.*, 1994)

M. chilensis (24-51%, Toro and Sastre, 1995), *A. purpuratus* (66.7%, Toro *et al.*, 1995) but less effective with those reported for *M. edulis* (97.4%, using a wide range for 3N number of chromosomes, 35-48, Yamamoto and Sugawara,1988). The highest percentages of triploidy (31.0-74.3%) obtained in this study by blocking meiosis II treating eggs with heat shock in this study is comparable to those reported for *M. galloprovincialis* (78.1-84.2%, Scarpa *et al.*, 1994), *M.chilensis* (15-31%, Toro and Sastre, 1995), *C. gigas* (13-35%, Quillet and Panelay, 1986). Quillet and Panelay (1986) and Yamamoto and Sugawara (1988) demonstrated that the effects of heat shock could be increased by extending the duration of treatment. Chemical treatment with CB appears to be temperature-dependent in inducing triploidy in bivalves. Thus, raising the temperature during treatment with CB in *C. gigas* increased the success of triploid induction and lower temperatures would reduce the efficiency of CB (Dowining and Allen, 1987). Yamamoto *et al.* (1990) reported increased yields of triploid *C. gigas* using a combination of heat shock (32°C) and caffeine (10 m*M*), compared to heat shock alone. In contrast to this , the combined chemical and thermal treatments were not more effective than the thermal treatment alone for producing triploid larvae (Scarpa *et al.*, 1994).

As it was previously reported by Wada *et al.* (1989) that cold shock (6.0-6.5°C) was effective in inducing triploidy (45-52%) in *P. fucata martensii*, in the present study lower temperature of 6±1°C was employed for 15 min induced polyploidy on par with heat shock treatment at 35°C for 10 minutes (Experiment 3). The results from the Experiment 3 (14.3-49.3% triploidy) established the effectiveness of cold shock for inducing triploidy in *P. fucata*. This observation affirmed that cold shock induce triploid in *P. fucata* in various other bivalves like *M. edulis* (Yamamoto and Sugawara, 1988), *O.cucullata*

(23.5-36.7%, Zeng *et al.*, 1994) and *O. gigas* (68%, Yu, 1994). However, in the present study, heat shock was observed to be more effective than cold shock as already reported by Yamamoto and Sugawara (1988).

The usefulness of triploidy in the aquaculture of bivalves depends particularly on the ease and success of inducing it. The methods, other than thermal shock used to induce triploidy in bivalves included chemical shock with CB and 6-DMAP, pressure shock and electrofusion method. Although, CB is costly, carcinogenic and having negative effect on larval survival (Stanley *et al.*, 1981; Allen *et al.*, 1982; and Tabarini, 1984), and its effectiveness on polyploidy is more in inducing triploidy than heat shock in *C. virginica* (75%, Stanley *et al.*, 1981 and 84%, Barber *et al.*, 1992), *C. gigas* (88±9%, Downing and Allen, 1987 and 90%, Desrosiers *et al.*, 1993), *O. cucullata* (87.5%, Zeng *et al.*, 1994), *A. irradians* (94%, Tabarini, 1984), *M. galloprovincialis* (86%, Scarpa *et al.*, 1994), *R. decussatus* (95%, Gerard *et al.*, 1994b), *T. dorsatus* (56-85%, Nell *et al.*, 1995) and *P. fucata martensii* (100%, Wada *et al.*, 1989). Thermal shock arrests all development, only in those eggs which are at a vulnerable stage of cell division at the time of shock, will be affected by the shock treatment while CB does not appear to interrupt development in the same way and eggs will continue to develop, during treatment, until they reach a vulnerable stage of cell division (review by Beaument and Fairbrother, 1991). However, low percentages of triploidy were also reported by Guo *et al.* (1992). This may be due to the carcinogenic effect of CB and which might have altered the physiology of the developing embryo leads to deformity.

An alternative drug, 6-DMAP has also been reported to induce triploidy (Desrosiers *et al.*, 1993; Gerard *et al.*, 1994a and Nell *et al.*, 1996). Pressure

shocks were found to be least efficient (about 60%) (Chaiton and Allen, 1985; Anderson and Wallace, 1996) while average yields of heat shocks or CB treatments are similar and often higher than 70% (Stanley *et al.*, 1981). However, Shen *et al.* (1993) have reported 76% triploidy in *P. fucata martensii* due to pressure shock which is comparable to the results obtained in the present study.

5.3. Tetraploidy inducement

Highest percentage of tetraploidy (40.6%) obtained by cold shock at 6°C and by heat shock (46.6%) at 35°C reported in the present study by suppression of first cleavage are comparable to those reported for the bivalves, *C. gigas* (30-45%, Guo *et al.*, 1994) and *C. rivularis* (28-35.8%, Shoubai *et al.*, 1994), *O.rivularis* (28-30%, Gendreau and Grizel, 1990). Cadoret (1992) induced 26% tetraploidy by electrofusion method. Such slight variations could be partly explained by differences in the procedures adopted among the laboratories gamete quality and other variations related to the methods themselves.

5.4. Larval development

Although not investigated in this study, heat shock (38°C) has been reported to affect early larval development and survival. Quillet and Panelay (1986) reported that higher temperature (38°C) reduced survival rate of larvae. Yamamoto and Sugawara (1988) could not get sufficient larvae to make chromosome spreads due to very high temperature (38°C) induced mortality. Eventhough, 38°C groups induced ploidy as that of 35°C groups, the abnormal larvae were found in higher numbers. The abnormal larvae were identified by its retarded shape and constant circular movements.

Thus, the present study advocate the utilization of thermal shock (upto 35°C) for the effective production of polyploid pearl oyster, *P. fucata* with minimum mortality. Such higher survival coupled with enhanced rate of polyploid oyster will pave the wave for the commercial production of cultured pearls.

SUMMARY

6. SUMMARY

In the present study, inducement of polyploidy in *P. fucata* (G.) by thermal shock was investigated.

1. Matured pearl oysters were collected from the natural pearl banks of Gulf of Mannar, off Tuticorin.

2. The ripe oysters were thermally (34 ± 1°C) induced to spawn in the hatchery. Eggs and sperm were collected separately and kept at filtered sea water where the water temperature was maintained at 24 ± 1°C.

3. Eggs were fertilized with sperm at 24 ± 1°C at known time.

4. Polyploidy was induced by thermal shock and the effect of initiation time and temperature (6,30,32,35 & 38°C) of treatment on triploidy and tetraploidy induction was studied.

5. Most of the eggs at 24 ± 1°C completed the first meiosis 15 minutes after fertilization and second meiosis at 25 minutes after fertilization.

6. The ploidy level of all treated groups and controls was determined by direct chromosome counts.

7. Triploid embryos were found in all treated groups. Also, aneuploids were observed in all the treatments.

8. The optimal thermal shock procedure for triploidy induction was the heat shock (35°C for 10 min) applied 10 min (meiosis I) and 20 min (meiosis II) after fertilization, resulted in highest triploidy percentages of 69.3 ± 2.96%

and 74.3± 2.33 % respectively. These were significantly higher ($F_{(4,3)}$= 5.2118, P>0.05) than all other thermal shock treatments except 38°C (64.6%) groups.

9. The highest tetraploidy percentage (46.6±1.66%) was also achieved by heat shock (35°C for 10 minutes) applied 50 min after fertilization. This is significantly higher ($F_{(4,10)}$ = 17.7883, P<0.01) than all other treatments except , the groups treated at 7 and 38°C.

10. The effect of heat shock and cold shock for polyploidy induction was statistically compared and it is found that heat shock was significantly higher (t=6.1061, P<0.05) than cold shock for triploidy induction and not significant (t=1.59, P>0.05) for tetraploid induction.

11. From this study, it became evident that triploid pearl oyster can easily be produced by thermal shock and this method is highly recommended for commercial production because of the absence of the need for costly special chemicals and sophisticated equipments.

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